Molecular cloning and gene expression analysis in aquaculture science: a review focusing on respiration and immune responses in

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REVIEWS

Molecular cloning and gene expression analysis in aquaculture science: a review focusing on respiration and immune responses in European sea bass (*Dicentrarchus labrax*)

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Abstract Although fish farming has been practiced for 4,000 years, aquaculture research dates back only to about 1,870, whereas molecular techniques, in addition to the more traditional methods of biotechnology, were introduced only recently. Contemporary genomic approaches (often adapted from human or medical research) such as cDNA cloning and sequencing, cDNA microarray/expression analysis, and functional genomics, combined with improvements in transgenic technologies, have enhanced possibilities for aquacultural biotechnologists for improving fish growth rates and increasing resistance to pathogens and stressors. Although genomic technologies in fish have been applied primarily to model organisms, such as zebrafish (*Danio rerio*), fugu or pufferfish (*Tetraodon nigroviridis*), and medaka (*Oryzias latipes*), many teleosts of interest for biological research and with potential application in aquaculture have unique physiological characteristics that cannot be directly investigated from the study of small laboratory fish models. As a consequence, large-scale genomic research studies are increasingly being applied to farmed species of economic relevance, such as farmed rainbow trout, Atlantic salmon, tilapia, catfish, and sea bass. Accordingly, we describe the utilization of molecular cloning and gene expression analysis in cultured European sea bass (*Dicentrarchus labrax*) to generate "transcriptome-focused" information which may enable a better understanding of the transcriptional programs that underlie fish respiratory physiology and immune response pathways.

Keywords Aquaculture biotechnology · Functional genomics · Hypoxic stress biomarkers · Hypercapnia · Molecular cloning · Gene expression · Fish innate immune system

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Introduction

Molecular cloning is one of the key genomic technologies that have made considerable advances possible in biology over the past 50 years. Molecular cloning

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refers to the procedure of isolating a defined DNA sequence and obtaining identical copies of that sequence in an organism. Most of our knowledge about gene transcripts and proteins has been derived from the ability to prepare complementary DNA (cDNA) from RNA and to clone it into cDNA libraries (for a review, see Harbers 2008).

Techniques for cDNA cloning improved by the mid-1970s, after the discovery of reverse transcriptase, which made it possible to enzymatically convert RNA into double-stranded cDNA (Baltimore 1970; Temin and Mizutani 1970). With efficient methods for cDNA synthesis, biologists then began to clone and study the structure and function of individual genes, thus obtaining complete sequence information and the possibility of designing and executing experiments to query different aspects of a gene's function (Galas and McCormack 2003; Harbers 2008). In human genetics, the most successful application of DNA cloning was the isolation of specific genes and identification of single or multiple mutations that could be causally related to a disease phenotype in humans. Many of these "disease genes" were isolated in the 1980s and early 1990s, including the cystic fibrosis gene, the muscular dystrophy gene, and others, and the process continues today.

In aquaculture, molecular techniques, in addition to the more traditional methods of biotechnology, were introduced only recently, although fish farming has been practiced for 4,000 years, and aquaculture research dates back to about 1,870. Contemporary genomic approaches (often adapted from human or biomedical research), such as cDNA cloning and sequencing, cDNA microarray/expression analysis, and functional genomics, combined with improvements in transgenic technologies, has opened up new possibilities for aquacultural biotechnologists for improving fish growth rates, increasing resistance to pathogens and stressors, improving quality of the broodstocks, and creating the opportunity to make new or different products by altering their genetic makeup (Melamed et al. 2002; Adams and Thompson 2006; Overturf 2010).

Aquaculture is currently contributing almost half of the fish consumed by the human population worldwide [FAO (Food and Agriculture Organization of the United Nations) 2009]. With the need to feed a growing population, the worldwide decline in ocean fisheries stocks and an interest in healthy foods, aquaculture has acquired great importance and is growing more rapidly than other animal food-producing sectors (FAO 2009). Unlike traditional agriculture, where a limited number of species are under study, more than 150 diverse fish species (FAO 1996) are being cultured and studied in multiple environments in aquaculture. This number is increasing yearly, the emphasis in recent years being to develop technologies to reproduce and grow marine species with high market value.

The increasing importance of aquaculture is also reflected in the increasing number of genomic studies performed on commercial fish species in recent years. Although genomic technologies in fish have been applied primarily to model organisms, such as zebrafish (*D. rerio*), fugu (*Takifugu rubripes*) or pufferfish (*T. nigroviridis*), and medaka (*O. latipes*), many teleosts of interest for biological research and with potential application in aquaculture have unique physiological characteristics (large adult size with considerable muscle production) that cannot be directly investigated from the study of small laboratory fish models. As a consequence, large-scale genomic research studies are increasingly being applied to farmed and wild fish species of economic relevance, such as farmed rainbow trout, Atlantic salmon, tilapia, catfish, and Atlantic cod.

However, the lack of existing genetic information has been a major drawback for more general application of currently available genomic technologies on most aquaculture fish species. At present, the complete sequence of the nuclear genome is known for only five fish species (*D. rerio, O. latipes, T. rubripes, T. nigroviridis,* and *Gasterosteus aculeatus*), and funding has been obtained for whole genome sequencing of at least four commercially important aquaculture fish species (Atlantic salmon, rainbow trout, tilapia, and Channel catfish). Two of these genome projects, for rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), are due to be completed by 2012 (Oleksiak 2010).

Although nuclear genome projects are lacking for most fish species, a wealth of sequence data exists for many more fish species. This is due to the fact that, in parallel with the feasibility studies leading up to the genome sequencing projects, a shortcut to the core of the problem has been developed: characterizing only the expressed part of the genome, the messenger RNAs (mRNAs), by sequencing randomly selected complementary DNA (cDNA) clones (Carninci 2007).

Table 1 Sea bass cDNA sequences characterized and deposited in GenBank database

Gene name	GeneBank accession number	Physiological function of the encoded protein
GR (glucocorticoid receptor)	AY549305	The receptor to which glucocorticoids (cortisol) bind
BDNF (brain-derived neurotrophic factor)	FJ711591	Supports the survival of CNS existing neurons, and encourage the growth and differentiation of new neurons and synapses
HSP70 (heat shock protein)	AY423555	Involved in the folding and unfolding of other proteins. Their expression is increased when cells are exposed to elevated temperatures or other stress
HSP90	AY395632	
HMGCoR (3-hydroxy-3-methylglutaryl-coenzyme A reductase)	AY424801	The rate-controlling enzyme of the mevalonate pathway, the metabolic pathway that produces cholesterol
C Fos (proto-oncogene protein)	DQ838581	Immediate early gene. Indirect marker of neuronal activity
NHE-1 (Na +/H + exchanger 1)	FJ711591	Maintenance of the body's salt and water homeostasis as well as intracellular pH regulation
HIF-1α (hypoxia inducible factor-1 alpha)	DQ171936	Regulates genes involved in response to hypoxia
BAL (bile-salt activated lipase)	EU647691	Enzyme produced by the pancreas that aids in the digestion of fats
Glut 2 (glucose-transporter)	EF014277	Facilitative sodium-independent glucose transporter that transports both D-glucose and D-fructose
Cathepsin L	FJ807676	Lysosomal cysteine proteinase that plays a major role in intracellular protein catabolism
Calpain 1	FJ821591	Calcium-dependent, non-lysosomal cysteine protease (proteolytic enzyme)
Ghrelin	DQ665912	Potent, peripherally active, appetite-stimulating hormone predominantly secreted by the stomach
Progastricsin (pepsinogen C)	EF690286	Inactive precursor of pepsin, gastric enzyme in charge of the initial and partial hydrolysis of the dietary proteins
FaDsD (fatty acid Δ6-desaturase)	EU647692	Enzyme involved in the biosynthesis of essential fatty acids
Lipin 1	EU644089	It has an enzymatic role in triglyceride and phospholipid biosynthesis, and also acts as an inducible transcriptional coactivator in conjunction with peroxisome proliferator-activated receptor gamma (PPAR gamma) and PPAR alpha. Through these activities, lipin-1, influences lipid metabolism and glucose homeostasis in diverse tissues including adipose tissue, skeletal muscle, and liver
IGF1 (insulin like growth factor-I)	AY800248	Ubiquitous small peptide, well known for stimulating many anabolic responses on a variety of target cells in both endocrine and autocrine/paracrine fashions
IGF2	AY839105	It is considered a major embryonic, foetal and early neonatal life growth factor
IGFBP-2 (IGF binding protein)	EU526670	The protein to which IGFs bind
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	AY863148	Enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. GAPDH has recently been implicated also in several non-metabolic processes, including transcription activation, initiation of apoptosis, and endoplasmic reticulum to Golgi vesicle shuttling
FGF6 (fibroblast growth factor)	AY831723	Member of the fibroblast growth factor family, in vivo moderator of critical phases of muscle development. Involved both in the proliferation and in the differentiation of the myogenic lineage by stimulating myoblast proliferation
14-3-3	DN832147	This highly conserved protein family is found in both plants and mammals. The encoded protein may play a role in linking mitogenic signaling and the cell cycle machinery

Table 1 continued

Gene name	GeneBank accession number	Physiological function of the encoded protein
MSTN (myostatin)	AY839106	A negative growth and differentiation factor which inhibits myoblast proliferation and plays important roles in the regulation of growth and development of many diverse tissues, including skeletal muscle
MyHC (myosin heavy chain)	DQ317302	Member of the family of ATP-dependent motor proteins, best known for their role in muscle contraction and their involvement in a wide range of other eukaryotic motility processes
β-actin	AY148350	One of the two non-muscle cytoskeletal actins which are highly conserved proteins that are involved in cell motility, structure and integrity
α- actin	FJ716131	A major constituent of the contractile apparatus
α- actinin	HM147821	An actin-binding protein with multiple roles in different cell types
HLP1 (Histone H2B- histone like protein1)	JN410660	Endogenous antimicrobial polypeptide (AMPP) directly and rapidly killing pathogens such as bacteria, fungi, parasites, and viruses. It is included within the realm of innate defenses. It was designated histone-like protein as it is closely related to histone H2B
HLP2 (Histone 1- Histone like protein 2)	JN410661	Endogenous AMPP
Hemoglobin β like protein	JN410659	Peptides derived from the respiratory protein hemoglobin, which have in recent years, been shown to be commonly linked to innate defense
Crystallin beta B1	AY971674	One of the dominant structural components of the vertebrate eye lens
PepT1 (oligopeptide transporter)	FJ237043	Integral plasma membrane protein responsible for the uptake of dietary di- and tri-peptides into cells. It transports peptides against a concentration gradient by coupling the movement of substrate across the membrane, with the movement of protons down an inwardly directed electrochemical proton gradient

These sequences, which are called expressed sequence tags (ESTs), represent a partial sequence of a much longer mRNA in the cell. They encode genes that are actively transcribed without intervening intron sequences and can thus be annotated to provide information about the ultimate function of the gene (Oleksiak 2010). Furthermore, generating an EST database represents an advantageous approach when funding is limited, as it can be realised very rapidly and at significantly less cost than other approaches (Wortheya and Myler 2005). As a consequence, large libraries of partial sequences of thousands of expressed genes already have been established. Large databases for EST sequences are available for many vertebrates, including fish species such as bluefin tuna (*Thunnus thynnus*), European perch (*Perca fluviatilis*), and European sea bass (*D.labrax*) (Rossi et al. 2007; Chini et al. 2006, 2008).

The European sea bass *D.labrax* L. (Moronidae, Perciformes, Teleostei), which represents the primary target species of our research, is a major fishery and aquaculture species on the Mediterranean and Atlantic coasts of Europe and North Africa. However, no representative of the Perciformes, the most advanced and diverse group of teleosts, has been fully sequenced, and genomic resources for this taxonomic group are relatively limited (Kuhl et al. 2010). The nuclear DNA content of European sea bass has been estimated at 1.55–1.58 pg (Peruzzi et al. 2005), approximately twice that of *T. rubripes* (Elgar et al. 1996), which despite the advances in sequencing technologies, still represents a large financial and logistic hurdle. However, the current sequencing techniques may solve this situation, as a complete genome can be sequenced quite fast.

As an alternative to genome sequencing for discovering genes, we have successfully implemented a process for identifying unknown (i.e., not present in a database) genes in this species, assuming that a computational database resource contains orthologous sequences (i.e. the same gene in different species) sequences from phylogenetically related species. Indeed, a rapid increase

in size and organismal representation of sequence databases suggested that we should be able to identify a sizable number of protein-encoding genes from any fish species according to their similarity to already known gene sequences. Following this similarity-driven molecular cloning and sequencing strategy (for details please see the following paragraphs), we isolated about forty (Table 1) full-length sea bass cDNAs (cDNAs having an open-reading frame or ORF) which encode some physiologically relevant proteins (Table 1) in this species (Terova et al. 2005, 2007, 2008, 2009a, b; Sangaletti et al. 2009; Rimoldi et al. 2009).

However, a DNA sequence does not itself reveal the functionality of a gene, but rather represents a portal to understanding gene and gene product roles and how these processes are modified and regulated (McLean and Craig 2006). Making use of the DNA sequence data to understand the function of genes and how they determine phenotypes is known as functional genomics. Our research in this field of molecular biology is focused on studies of natural variations of fish-specific transcripts over time (such as the organism's development) or space (such as its body regions), as well as on studies of the impact of different environmental stimuli and experimental treatments on gene expression levels. The goal of this research is to identify specific genes influenced, either positively or negatively, by experimental manipulations that might prove valuable as molecular biomarkers.

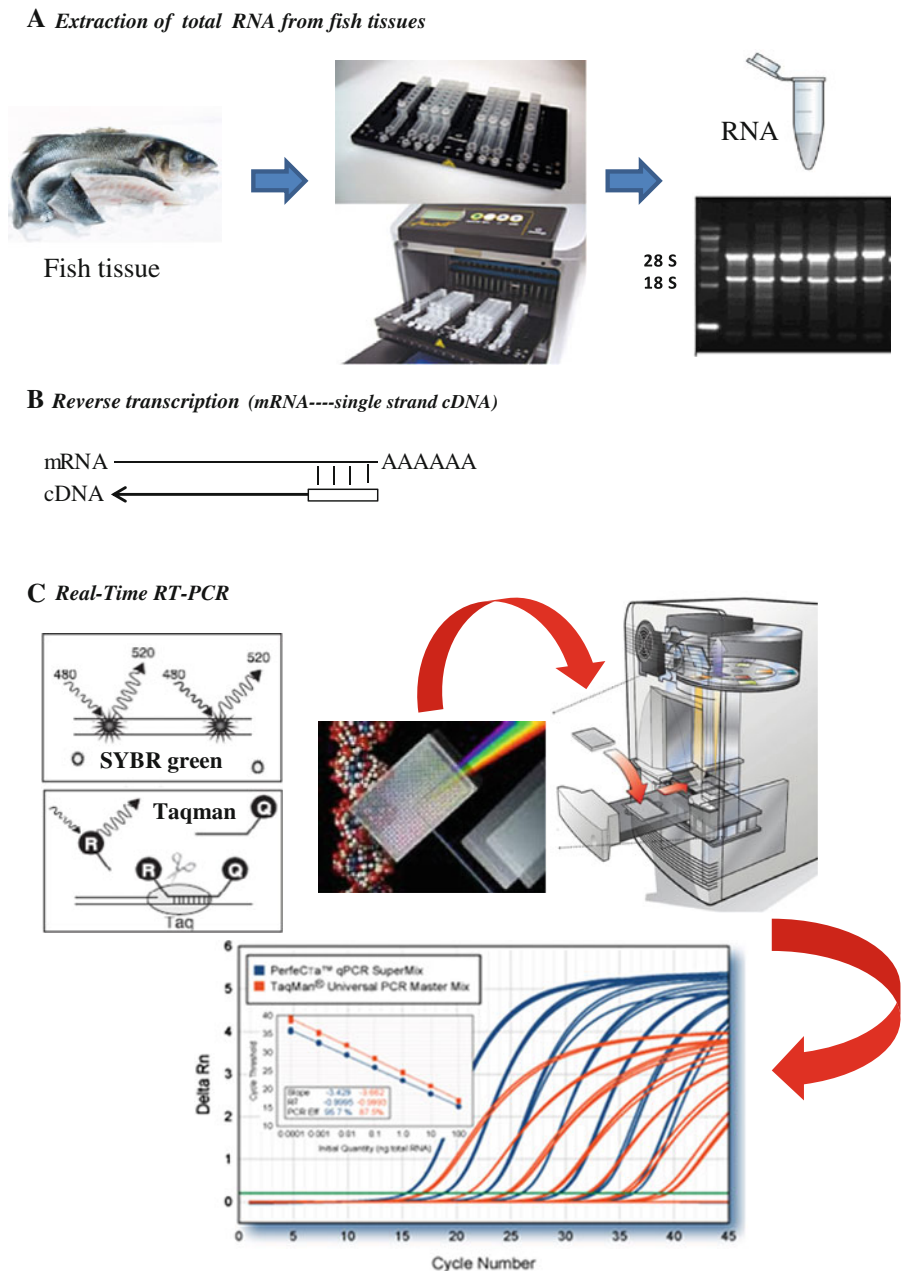
Technologies available to quantify gene expression range from single-gene, real-time PCR to high throughput methods such as cDNA macro- and microarrays, serial analysis of gene expression (SAGE), and massively parallel signature sequencing (MPSS). Real–Real Time quantitative Reverse Transcription PCR (Real-Time qRT-PCR), which is based on two sequential reactions—reverse transcription of the mRNA, followed by using the resulting cDNA as a PCR template—is considered as one of the most accurate methods for transcript evaluation (for a review, see Valasek and Repa 2005). qRT-PCR follows the general principle of the revolutionary method of PCR, developed by Mullis (1990) in the 1980s, which allows researchers to amplify specific pieces of DNA more than a billion-fold. As the name suggests, real-time PCR measures PCR amplification as it occurs. This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. In traditional PCR, results are collected after the reaction is complete, making it impossible to determine the starting concentration of nucleic acid. Figure 1 illustrates the principal steps of a qRT-PCR.

In aquaculture, the use of real-time PCR has recently expanded, in particular for detecting microbes, parasites, and genetically modified organisms. However, any need for fast and precise measurements of small amounts of nucleic acids in fish represents a potential niche for real-time PCR-based applications. As machines become faster, cheaper, smaller, and easier to use, more in-field applications for this technology in aquaculture are likely to be realized (Valasek and Repa 2005).

Accordingly, in the following paragraphs, we describe the utilization of reverse transcription real-time PCR in cultured fish to generate "transcriptome-focused" information which may enable us to better understand the transcriptional programs that underlie fish physiology and development.

Similarity-driven approach for molecular cloning and sequencing

At the beginning of our research on European sea bass (*D.labrax*), the cDNA sequences of genes listed in Table 1, such as glucocorticoid receptor (GR), insulin-like growth factor (IGF)-1, IGF-2, hypoxia-inducible factor (HIF-1alpha), glucose transporter-2 (Glut-2), Na^+/H^+ exchanger (NHE-1), oligopeptide transporter (PepT1) and so on, were unknown (not available in the GenBank database). We thus performed a BlastN search (http://www.ncbi.nml.nih.gov/BLAST/) on the complete, non-redundant GenBank nucleotide database for orthologues of these genes in other fishes/vertebrate species (Fig. 2). A ClustalW multiple nucleotide sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was then carried out on the sequences found and a strategy based on regions of strong nucleotide conservation was used to design the primers (Fig. 3). As an example, for the isolation of the GLUT2 cDNA coding sequence (Terova et al. 2009b), primer (sense and antisense) design was based on the alignment of three teleost GLUT2 coding sequences (Figs. 2, 3): *Gadus morhua*, (accession no. AY795481), *O. mykiss* (accession no. AF321816), and *Pseudopleuronectes americanus* (accession no. AY521663) coding sequences. These presented several conserved regions within the overall sequence where primers could be reasonably designed (Fig. 3).



After primer design, we proceeded with total RNA extraction from different fish tissues (it depends on the target gene) and first-strand cDNA synthesis. To perform PCR, an aliquot of the sea bass cDNA was amplified using a DNA polymerase and a set of the previously designed primers. An aliquot of each PCR reaction then was electrophoresed, and bands of the expected size were detected and cut from the gel. The PCR products from successful cDNA primer amplifications were subsequently cloned using a cloning vector system and then sequenced.

Different cDNA fragments of the target gene were obtained in this way. Then, by assembling the sequences of the partially overlapping clones, a partial coding sequence for the target gene was determined. As an example, in the case of GLUT2, two cDNA fragments were obtained using the designed primers.

◀**Fig. 1** Schematic representation of real time RT-PCR principal steps. **a** Total RNA is extracted from sea bass tissues using automated Maxwell® 16 System. This robust, automated RNA extraction system prevents cross-contamination by eliminating splashing, aerosols and drops from pipette tips during extraction, and provides fast automation of routine RNA extractions resulting in reproducible yields and purity. The integrity and relative quantity of RNA is checked by electrophoresis whereas the quantity of RNA is calculated using the absorbance at 260 nm. **b** After extraction, an aliquot of total RNA is reverse transcribed into cDNA using Moloney Murine Leukaemia virus reverse transcriptase as described in the M-MLV Reverse Transcriptase kit (Invitrogen). **c** Real-time quantification of target genes transcripts. Two types of chemistries are used to detect PCR products using real-time PCR instruments: SYBR green and Taqman®. Our approach relies on the use of gene specific Taqman® technology, and standard curve method for target mRNA quantification. The first step in this direction is the generation of in vitro transcribed standards of mRNAs. The mRNAs produced by in vitro transcription then are used as quantitative standards in the analysis of experimental samples. Real-time Assays-by-Design ^{SM} PCR primers and gene-specific fluorogenic probes were designed by Applied Biosystems. TaqMan® PCR is performed on a StepOne Real Time PCR System. Data from the Taqman® PCR runs are collected with StepOne^{TM} Software. CT (cycle threshold) values correspond to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values are used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA

Then, by assembling the sequences of these partially overlapping clones, a partial coding sequence (~1,500 bp) for sea bass GLUT2 was determined that shared a high sequence similarity with the GLUT2 proteins of various vertebrate species.

To isolate the complete ORF sequence, including the 5′- and 3′-end untranslated regions UTR), we used the rapid amplification of the cDNA ends (RACE) method (Yeku et al. 2009). The resulting amplification products then were electrophoresed, purified, cloned into a vector system, and sequenced.

Real time gene expression analysis

After cDNA sequence identification (all the identified sequences have been deposited in GenBank database), we proceeded with the real-time quantification of our target transcripts. Our approach relies on the standard curve method for target mRNA quantification. In this way, the number of each gene transcript copies could be quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of this gene. The first step in this direction is the generation of standards of mRNAs by in vitro-transcription.

The mRNAs produced by in vitro transcription then are used as quantitative standards in the analysis of experimental samples. Samples are analysed by using the Taqman® real-time RT-PCR method and One-step TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Italy).

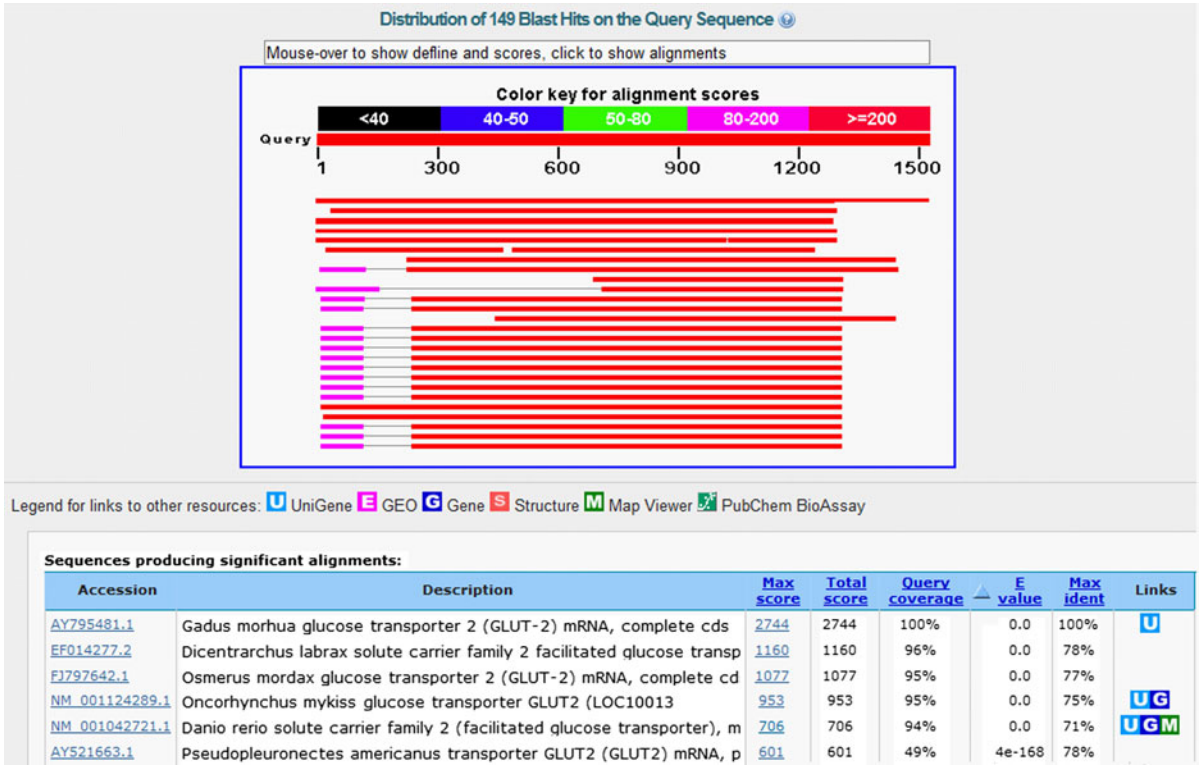
Molecular cloning and expression analysis of some genes involved in the response to aquatic hypoxia

At a practical level, respiration can be defined as the sum of processes by which the respiratory gases oxygen (O_2) and carbon dioxide (CO_2) pass from the environment to the tissues, and vice versa. Two important variables governing respiration are the concentrations of the respiratory gases and their partial pressures. In the vast majority of fish species, the main respiratory organ is the gill. The gills of fish are used to extract oxygen from the water and, in return, excrete carbon dioxide and toxic metabolic wastes, like ammonia and acid ions.

However, the environmental condition can reduce the availability of oxygen, causing hypoxia. Aquatic hypoxia is a common and frequent event, and fish often have to contend with hypoxic stress in order to survive in environments with low or variable oxygen levels. Acute decreases in water oxygen concentrations may occur in intensive fish farming as well, especially when fish are reared at high densities. In this respect, considerable attention has been paid to oxygen, as low ambient O_2 concentrations are known to affect growth, food intake and the physical status of fishes (Jobling 1994).

Another common event which, paradoxically, occurs frequently even in well-oxygenated water when fish have reduced their breathing rates, is hypercapnia (elevated blood CO_2 pressure due to an increasing of ambient free P_{CO_2}). In water-breathing animals, hypercapnia often develops under conditions related to intensive aquaculture with oxygenation and reuse of water. While acute episodes of elevated P_{CO_2} in water can be prevented by appropriate culture practices, it is quite difficult to monitor recurrent and chronic events.

Several studies have investigated the adaptive response of fish to low oxygen levels from a morphological/physiological point of view (Rinaldi et al. 2005); however,



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AY795481.1	Gadus morhua glucose transporter 2 (GLUT-2) mRNA, complete cds	2744	2744	100%	0.0	100%	U
EF014277.2	Dicentrarchus labrax solute carrier family 2 facilitated glucose transp	1160	1160	96%	0.0	78%	
FJ797642.1	Osmerus mordax glucose transporter 2 (GLUT-2) mRNA, complete cd	1077	1077	95%	0.0	77%	
NM_001124289.1	Oncorhynchus mykiss glucose transporter GLUT2 (LOC10013	953	953	95%	0.0	75%	UG
NM_001042721.1	Danio rerio solute carrier family 2 (facilitated glucose transporter), m	706	706	94%	0.0	71%	UGM
AY521663.1	Pseudopleuronectes americanus transporter GLUT2 (GLUT2) mRNA, p	601	601	49%	4e-168	78%	

Fig. 2 The results of a BLAST search for GLUT2 orthologous in teleost species

the molecular responses to hypoxia have not been extensively studied in fish, even though these animals are ideal models for the purpose. Understanding how hypoxia alters gene expression in fish likely will contribute to our knowledge of how vertebrates respond to hypoxia in general and illustrate the dynamic interactions between genes and environment (Ton et al. 2003). Accordingly, we used real-time PCR to examine the response at the transcriptomics level of *D. labrax* exposed to conditions of acute and chronic hypoxia.

Glucose transporters are membrane proteins active in the transport of hexoses such as glucose, galactose, and fructose across plasma membranes. They are divided into two structurally and functionally distinct families: sodium/glucose cotransporters (SGLTs) and facilitative sodium-independent glucose transporters (GLUTs) (Wood and Trayhurn 2003). SGLTs reside in the membranes of intestinal and kidney epithelial cells (Burant et al. 1991), whereas GLUTs can be found in most animal tissues.

GLUTs are known to be hypoxia-sensitive: expression of GLUT genes in mammals is regulated through hypoxia-inducible factor (HIF-1alpha), via its binding to the hypoxia-responsive elements present in the GLUT genes (Ebert et al. 1995). Increased expression of GLUT genes in hypoxic mammalian tissues has been associated with an enhanced glucose uptake rate to facilitate the supply of metabolic energy and to protect cells from hypoxic injury (Lin et al. 2000). Much less information is available on tissue expression patterns of GLUTs in fish exposed to hypoxia, and the mechanisms involved in the hypoxia responses of these genes are almost unknown.

At least 13 different GLUT isoforms have been identified thus far in different mammalian and avian tissues (Wood and Trayhurn 2003), each being the product of a separate gene. These integral membrane glycoproteins are characterized by the presence of 12 putative transmembrane-spanning domains with cytosolic amino and carboxyl termini (Pessin and Bell 1992). They display significant sequence homology, different affinities for glucose, different tissue-specific patterns of distribution, and may be subject to differential hormonal regulation (Joost et al. 2002). On the basis of sequence similarities and characteristic signature motifs, GLUT isoforms are categorized into class

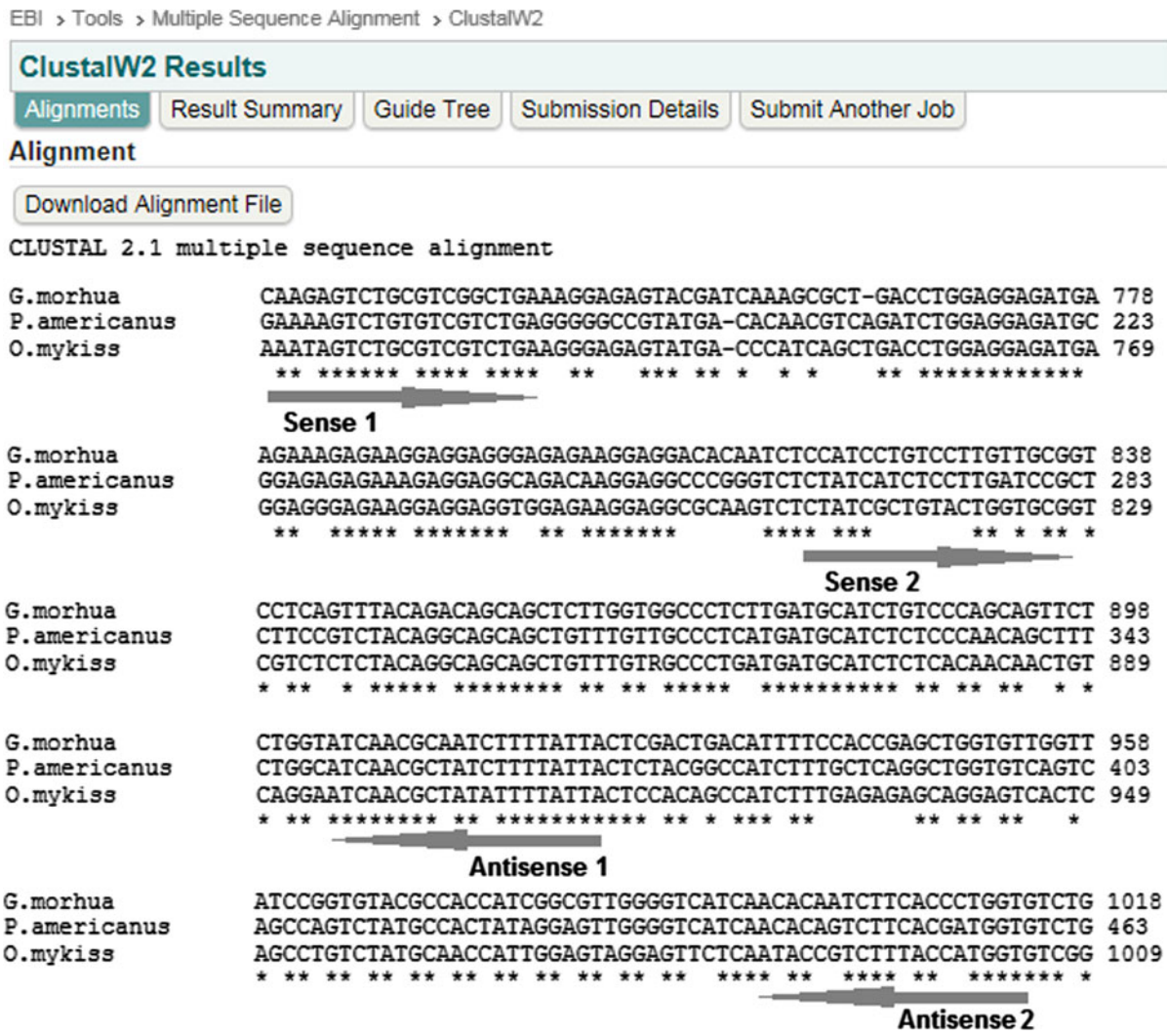


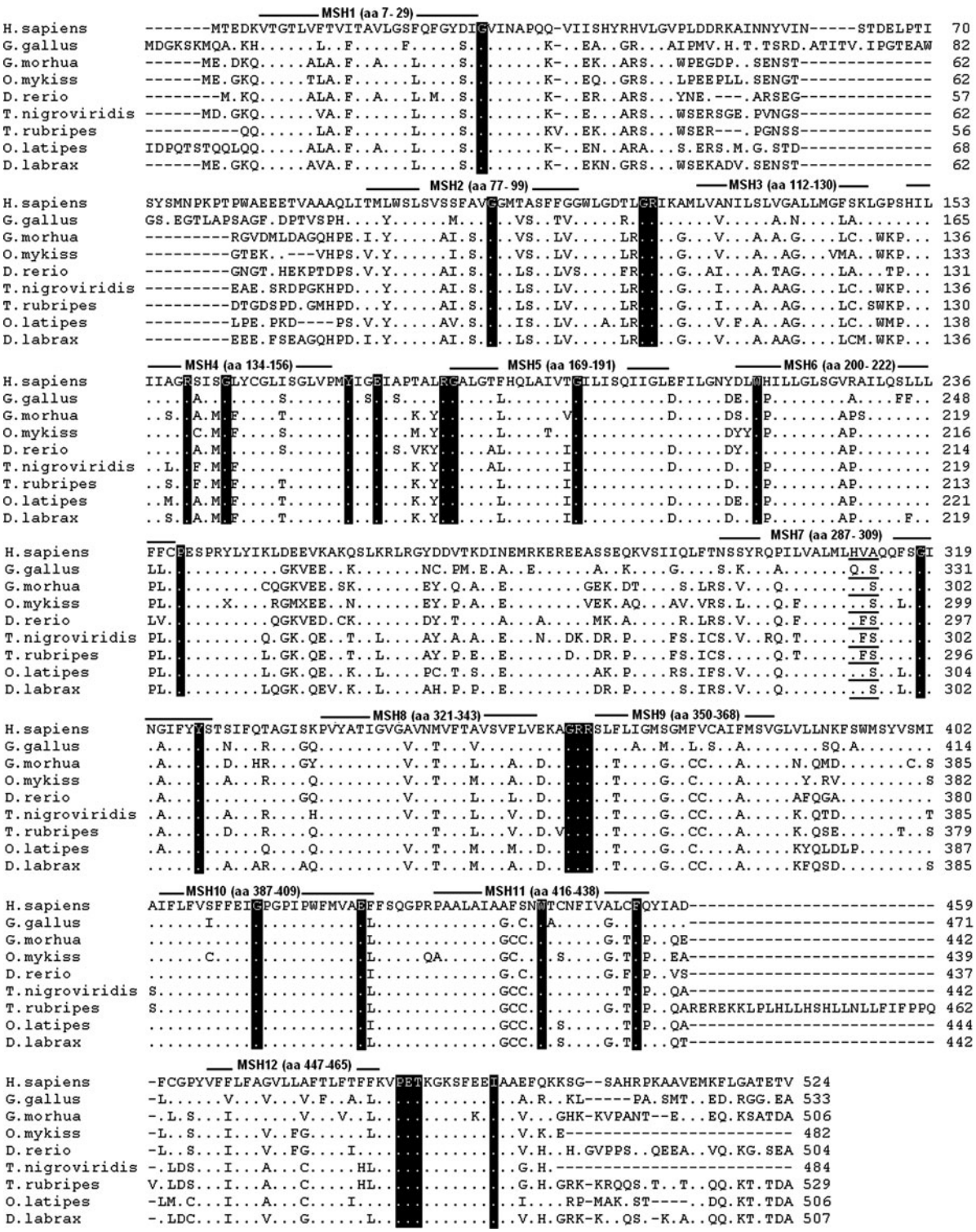
Fig. 3 ClustalW multiple GLUT2 nucleotide sequence alignment and primer design

I (GLUT1–4), class II (GLUT5, 7, 9, and 11), and class III (GLUT6, 8, 10, 12 and the myo-inositol transporter (HMIT1) (Joost et al. 2002; Wood et al. 2007).

The class I facilitative transporters have been thoroughly characterized in mammals. Given their high glucose specificity and the predominant expression in high energy demanding tissues, they have been the focus of several metabolic studies examining glucose and energy homeostasis (Katsumata et al. 1999).

In recent years, the four mammalian GLUT homologues belonging to class I also have been identified in fish: GLUT1 in tilapia, rainbow trout, common carp, and Atlantic cod (Wright et al. 1998; Teerijoki et al. 2000; Hall et al. 2004, 2006); GLUT2 in rainbow trout and Atlantic cod (Krasnov et al. 2001; Hall et al. 2006); GLUT3 in grass carp and Atlantic cod (Zhang et al. 2003); and GLUT4 in brown trout and Atlantic cod (Planas et al. 2000; Hall et al. 2006). The presence of mammalian GLUT homologues in fish has kindled an interest in studying their transcriptional regulation, not only because of the importance of glucose uptake in the overall energetic and regulation of carbohydrate metabolism, but also since many fish species face either acute or chronic low oxygen challenges.

Accordingly, we focused our attention on GLUT-2, which is the major glucose transporter in hepatocytes and in β-cells of pancreatic islets in mammals. GLUT-2 transports both D-glucose and D-fructose and its


MSH1 (aa 7-29)
MSH2 (aa 77-99)
MSH3 (aa 112-130)
MSH4 (aa 134-156)
MSH5 (aa 169-191)
MSH6 (aa 200-222)
MSH7 (aa 287-309)
MSH8 (aa 321-343)
MSH9 (aa 350-368)
MSH10 (aa 387-409)
MSH11 (aa 416-438)
MSH12 (aa 447-465)
H.sapiens
G.gallus
G.morhua
O.mykiss
D.rerio
T.nigroviridis
T.rubripes
O.latipes
D.labrax

◀ **Fig. 4** Alignment of the deduced aminoacid sequence of seabass (*D.labrax*) GLUT2 (accession no. ABJ98775) with the GLUT2 related protein of *H.sapiens* (accession no. NP_000331), *G.gallus* (accession no. NP_997061), *G. morhua* (accession no. AAV63984), *O. mykiss* (accession no. NP_001117761), *D. rerio* (accession no. CAQ15265), *T. nigroviridis* (accession no. CAF95896), *O. latipes* (ENSORLP 00000014512 http://www.ensemble.org/Oryzias_latipes) and *T. rubripes* (Joint Genome Institute (JGI) database accession no. 589792). Amino acids are designated by *single-letter* codes and are *numbered* to the *right side*. *Dots* indicate conserved residues compared to human GLUT2. *Dashes* indicate gaps introduced to facilitate alignment. The *numbered straight lines* on *top* of the sequence alignments give the positions of the membrane spanning helices (MSH) in sea bass, predicted using the THMM program (http://www.cbs.dtu.dk/services/). Residues that are *highlighted* by a *black background* represent absolutely conserved aminoacids in all members of the extended GLUT family (modified from Terova et al. 2009a)

half-maximal saturation constant, Km, is ~30 mM, making it a low-affinity and high-capacity hexoses transporter (Burant et al. 1991; McGowan et al. 1995; Leturque et al. 2005).

In the first step of our study, we isolated the full-length sea bass cDNA encoding GLUT2, which consists of 2,439 base pairs (bp) carrying a single open-reading frame that encompasses 1,524 bp of the coding region, 50 bp of the 5′ UTR, and 815 bp of the 3′ UTR, including a common (AATAAA) polyadenylation signal upstream of the poly(A) tail. Conceptual translation of the cDNA predicts a protein of 507 amino acids, (Fig. 4) which is similar to the GLUT2 of other fishes and higher vertebrate species as indicated by sequence analysis on the NCBI database. The sequence was deposited to the GenBank with accession number EF014277.

The transmembrane domains of the sea bass GLUT2 protein, predicted with the THMM program (http://www.cbs.dtu.dk/services/) are presented in Figs. 4, and 5. Sea bass GLUT2, like all GLUT isoforms (Mueckler et al. 1985) adopt a 12-transmembrane domain structure, with intracellular amino-and carboxyl-termini, an extra cellular loop between the first and second transmembrane segments, and a large intracellular loop between the transmembrane segments 6 and 7. The predicted sea bass GLUT2 amino acid sequence reveals also several functionally important sequence motifs described in all known members of the GLUT protein family (Mueckler et al. 1985; Sato and Mueckler 1999; Hall et al. 2004, 2006).

In the second step, we monitored the dynamic changes of GLUT-2 mRNA copy number in response to acute and chronic hypoxic stress conditions (Terova et al. 2009b). For the experiment, sea bass (*D.labrax*) were stocked into long indoor tanks and allowed to acclimate. After the acclimation period, for the chronic hypoxia exposure, fish were transferred into each of three experimental tanks connected to a recirculation system and allowed to acclimate for some other days. Then one of the tanks (control) was maintained under normoxic conditions, the second one under moderate hypoxic, and the third tank under hyperoxic conditions.

Fish from each of the three groups were sampled at the start of the experiment (day 0), and then after continuous exposure for 24 h, 48 h, 5 days, and 15 days to the respective experimental conditions. After 15 days of hypoxia or hyperoxia exposure, the DO levels in the two tanks were adjusted back to normal, saturated levels and, after a 24-h recovery period, the remaining fish were sampled from each tank. For the molecular biology analysis, brain, heart, liver, kidney, spleen, and muscle were isolated from each animal.

For the acute-hypoxia exposure, fish from the previously described stock were transferred into each of two 100 L tanks connected to a recirculation system and after 5 days of acclimation were exposed to severe hypoxia. The hypoxic DO value was chosen on the basis of our previous observations on sea bass.

Fish from the first tank were sampled 4 h after the target level of DO was achieved, immediately before they became close to death, whereas fish from the second tank were at the same moment, immediately re-oxygenated normally for 24 h and sampled at the end of the recovery period.

The Real-Time RT-PCR analysis revealed that GLUT2 transcription is significantly induced by chronic and acute hypoxic conditions. In sea bass subjected to chronic hypoxic conditions there were high levels of GLUT2 gene expression in intestine and liver (Fig. 6), lower levels in other tissues such as brain, kidney, heart, and muscle, and negligible expression levels in spleen.

The tissue distribution and relative abundance of GLUT2 in sea bass differed from those of other GLUTs described in fish. Hall et al. (2004, 2006) described a GLUT1 expressed in at least 10 tissue types of Atlantic cod (*G. morhua*). The same ubiquitous distribution for GLUT1 was previously found by Teerijoki et al. (2000) in rainbow trout (*O. mykiss*).

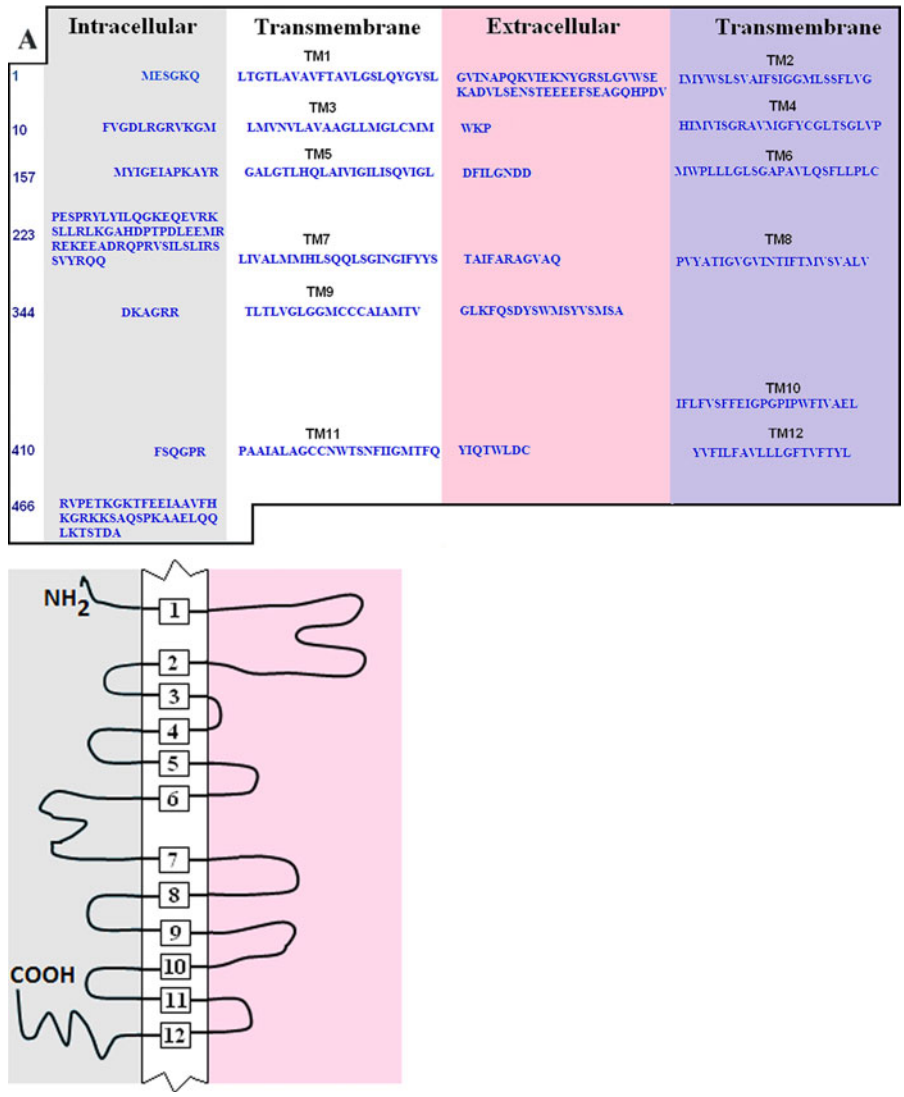


Fig. 5 Membrane topology of glucose transporter 2 (GLUT2) protein predicted with the THMM program (http://www.cbs.dtu.dk/services/). The protein contains 12 transmembrane domains, with the N-terminal and C-terminal ends in the cytosol. Based on the analysis of chimeric transporters derived from GLUT2 and GLUT3, transmembrane domains 7–8 provide the ability for fructose transport, whereas the transmembrane 9–12, which are GLUT2 specific, are believed to be responsible for the relatively low affinity to glucose. Transmembrane segments 9–12 are part of the pore through which glucose traverses the membrane

Furthermore, GLUT1 was predominantly expressed in the heart in rainbow trout, with low expression in other tissues. This finding was consistent with the abundance of this transcript in Atlantic cod heart (Hall et al. 2004, 2006) and detection of GLUT1 protein in the heart in tilapia (Wright et al., 1998). Planas et al. (2000) and Capilla et al. (2002) described a ubiquitously expressed GLUT4 from brown trout (*Salmo trutta*), with abundant transcription in skeletal muscle, gill, and kidney, but poor expression in heart, liver, and brain. GLUT4 levels were much higher than GLUT1 levels in red and white muscle of rainbow trout and, at least in red muscle, GLUT4 is insulin sensitive (Capilla et al. 2002). More recently, a GLUT3 was cloned and sequenced from grass carp (*Ctenopharyngodon idellus*) that is most abundant in kidney, with lower levels present in heart, brain, gill, liver, and muscle (Zhang et al. 2003).

The absolute amounts of mRNA for GLUT2 in the European sea bass liver in response to chronic hypoxic stress as estimated by real-time PCR are presented in Fig. 7a, b. These data demonstrate that GLUT2 expression is induced by hypoxia. At a hypoxic stress level of 4.3 mg/L DO and up to 48-h exposure, GLUT2 mRNA levels were the same as those found in normoxia-held fish (Fig. 7a), whereas they were significantly increased after 5 days of exposure. Longer exposure to hypoxia (15 days) contributed to a further increase in the GLUT2 mRNA copy number. Recovery for 24 h decreased the GLUT2 mRNA levels compared to the previous time

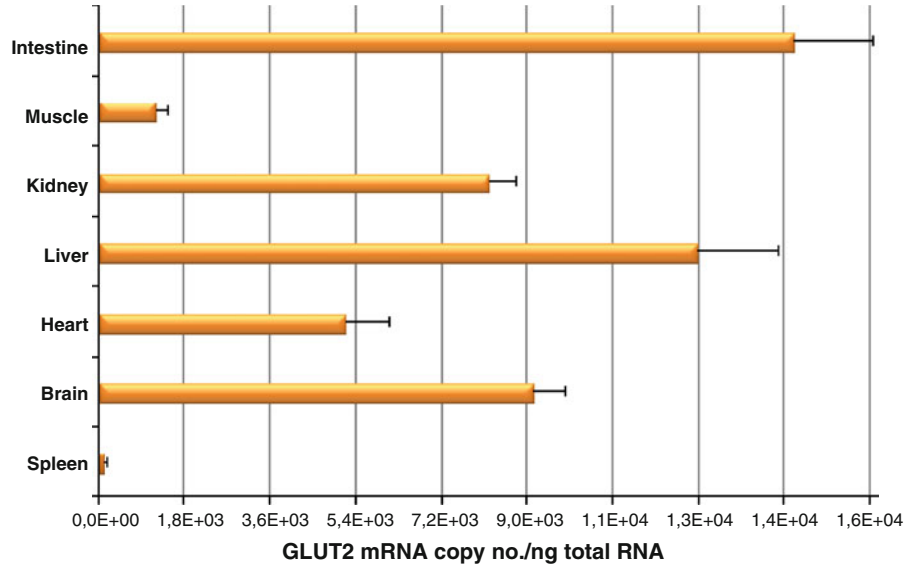


Fig. 6 Tissue distribution of GLUT2 mRNA in sea bass as determined by Real-Time quantitative PCR. Fish were sampled after 15 days of continuous exposure to the chronic hypoxic conditions (DO, 4.3 ± 0 mg/L, 51 % of saturation). GLUT2 copy number was normalized as a ratio to 100 ng total RNA. The means of five animals in each group are shown. *Bars* indicate standard error of the mean (modified from Terova et al. 2009a)

point tested (15 days of hypoxia), but they did not return to control values within 24 h of exposure to normoxic conditions. Severe hypoxic stress (DO 1.9 mg/L) for 4 h dramatically increased the number of GLUT2 transcripts in the liver (Fig. 7b). Subsequently, 24 h after recovery, the GLUT2 mRNA levels decreased compared to the previous time point tested (4 h hypoxia), remaining still significantly higher than the control values.

The obtained data could suggest that the GLUT2 isoform plays a role in the hypoxia-induced stimulation of glucose transport, which is required to fuel an elevated ATP demand supported by anaerobic metabolism. However, we do not know if the mRNA profile is consistent with the functional protein levels, and our hypothesis has to be confirmed by further investigations.

Molecular cloning and expression analysis of genes involved in the response to aquatic hypercapnia

Hypercapnia (increased blood CO_2 pressure, due to an increase of aquatic P_{CO_2}), is a common event under conditions of intensive aquaculture, and in fish a complex set of physiological and biochemical alterations are employed to cope with this environmental stress. Available data regarding the effects of CO_2 on fish show that hypercapnia negatively affects many vital physiological functions in exposed animals, e.g., respiration, circulation, central nervous system function, growth, behaviour, and metabolism. An increase in relative breathing amplitude was observed in *D. rerio* under hypercapnic conditions (Vulsevic et al. 2006), whereas in *Cyprinus carpio* the ventilation frequency significantly increased at high P_{CO_2} (Soncini and Glass 2000). The exposure of *Seriola quinqueradiata* to 5 % CO_2 (38 mmHg) resulted in a significant fall in cardiac output and stroke volume and in a rise in blood pressure (Lee et al. 2003). Söderström and Nilsson (2000) noted an increase in cerebral blood flow velocity and in dorsal aortic blood pressure in rainbow trout (*O. mykiss*) exposed to a P_{CO_2} of 22.5 mm Hg. In *S.salar*, hypercapnia caused a reduction in the condition factor (K) due to reduced feed intake (Fivelstad et al. 2003), and a decline in feed intake due to an increase of CO_2 concentration was also found in sea bass by our group.

The principal response to hypercapnia in fish is similar in freshwater and marine teleosts: CO_2 diffuses into the blood across the gills, causing a very rapid reduction of plasma pH and a decline in O_2 affinity of hemoglobin (Heisler 1984; Evans et al. 2005). However, plasma pH starts to recover through compensatory elevation of plasma bicarbonate (HCO_3^-) and Na^+ concentrations (Grøttum and Sigholt 1996) in conjunction with equimolar excretion of chloride (Heisler 1986) and H^+ to maintain electroneutrality (Cameron 1985). In fishes, more than 90 % of all acid–base equivalent ion transport processes occur across the gill epithelium and kidney (Heisler 1986; Perry and

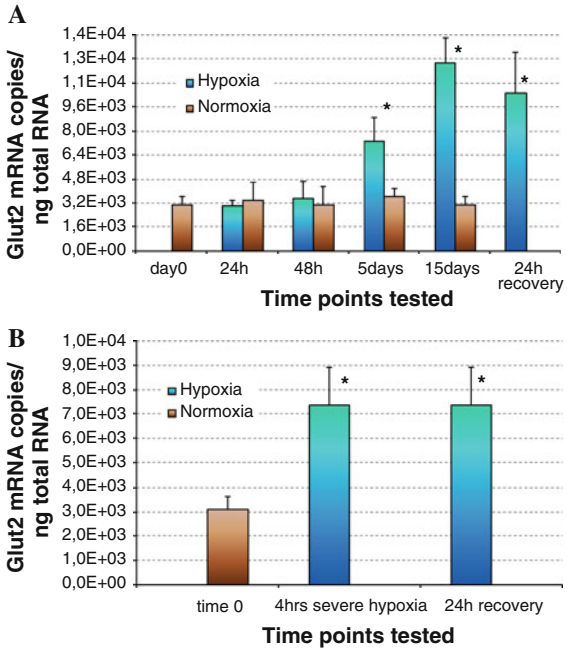


Fig. 7 **a** Expression levels of GLUT2 measured by real-time PCR in *D. labrax* liver in the course of (**a**) chronic hypoxia exposure (DO, 4.3 ± 0.8 mg/L, 51 % of saturation). Fish were sampled at the start of the experiment (day 0), and then after continuous exposure for 24 h, 48 h, 5, and 15 days to the hypoxia conditions. After 15 days of hypoxia the DO levels were adjusted back to normoxia levels and fish were sampled after 24 h of recovery. **b** Expression levels of GLUT2 measured by real-time PCR in *D. labrax* liver in the course of the acute hypoxia exposure. Fish were sampled after continuous exposure for 4 h, to severe hypoxia conditions (DO, 1.9 ± 0.2 mg/L). After 4 h of hypoxia the DO levels were adjusted back to normoxia levels and fish were sampled after 24 h of recovery. GLUT2 mRNA copy number was normalized as a ratio to 100 ng total RNA. The means of five animals in each group are shown. *Bars* indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). *Asterisks* indicate significantly different means from controls, for each time point tested ($P < 0.05$) (modified from Terova et al. 2009a)

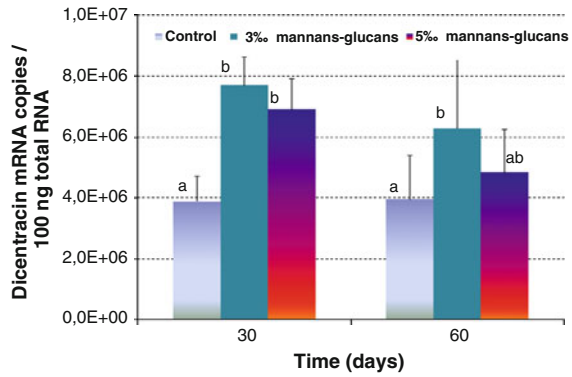


Fig. 8 Expression levels of dicentracin measured by real-time PCR in *D. labrax* head kidney in the course of the experiment. Dicentracin mRNA copy number was normalized as a ratio to 100 ng total RNA. Fish were sampled after 30 and 60 days of mannans-glucans feeding at 3 and 5 ‰. The means of five animals in each group are shown. *Bars* indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). *Different letters* indicate significantly different means ($P < 0.05$) (modified from Terova et al. 2009b)

Gilmour, 2006) and the best-known transmembrane transporters involved in this mechanism are the members of the Na^+/H^+ exchanger (NHE) family. In teleosts, the first NHE (β-NHE, which is homologous to NHE-1) was identified in the red blood cells of *O. mykiss* (Borgese et al. 1992), whereas other isoforms have been cloned from *Myoxocephalus octodecimspinosus* (Claiborne et al. 1999), *Myxine glutinosa* (Edwards et al. 2001), and *Fundulus heteroclitus* (Edwards et al. 2005). All fish Na^+/H^+ exchanger isoforms have been identified and cloned in gill, suggesting that NHEs are present as multiple isoforms in this tissue (Edwards et al. 2001; Hirata et al. 2003). Studies in different fish species have also indicated that NHE-protein expression changes as a result of acidosis (Edwards et al. 2001; Claiborne et al. 1999; Edwards et al. 2005).

Another gene expressed early in response to various stressful stimuli in mammalian brain, including CO_2/H^+ elevation (Sato et al. 1992), is c-Fos. Recently, c-Fos gene sequences have been reported also in some fish species, such as *T. nigroviridis*, *Carassius auratus*, *C.idellus*, *O. mykiss*, and *Rivulus marmoratus* (Matsuoka et al. 1998; Trower et al. 1996; Li et al. 2004). The lack of information regarding tissue expression patterns of c-Fos in fish exposed to hypercapnia conditions, especially regarding the participation of c-Fos and NHE genes in the molecular response to this stress, prompted our interest in cloning the c-Fos and NHE-1 genes in sea bass, and investigating the impact of acute and chronic exposure to hypercapnic conditions on both gene's mRNA copy number. The final aim of such an investigation was to find a valuable biomarker for CO_2 stress in farmed sea bass.

In the course of the study, we could isolate the complete cDNA sequence encoding sea bass c-Fos (GeneBank accession no. DQ838581) and a partial cDNA sequence encoding NHE-1 (GeneBank accession no. EU180587). We then analysed the expression

levels of both genes in the fish exposed to acute and chronic hypercapnic conditions.

Sea bass were stocked into long indoor tanks connected to a sea water recirculation system and allowed to acclimate before starting the trial. After the acclimation period, one of the tanks (control) was maintained under normal conditions until the end of the trial, whereas the other one was maintained under hypercapnic conditions. The hypercapnic condition was achieved by blowing pure CO_2 from a pressurized gas bottle directly into the tank.

Fish were sampled after 1 h of continuous exposure to the high hypercapnic conditions. For the molecular biology analysis, brain, gills, and kidney were isolated. The content of free CO_2 in the water then was decreased and other fish were sampled after continuous exposure for 3, 24, and 96 h to the hypercapnic conditions.

The c-Fos mRNA abundance in brain of sea bass increased sharply in response to acute hypercapnia (71.42 mg/L of free CO_2 for 1 h). It also was upregulated after the first 3 h of exposure to chronic hypercapnia (47.56 mg/L of free CO_2), but then decreased, reaching the control values after 96 h of exposure. The same pattern of expression for c-Fos gene also was observed in higher vertebrates such as rat and mice. In the neuronal cells of these animals, the c-Fos transcripts were upregulated within minutes after hypercapnic stimulation. The c-Fos protein expression also was upregulated after 30 min of exposure to the CO_2 stimulus, but decreased after a few hours of hypercapnic exposure (Tankersley et al. 2002; Pete et al. 2002; Shimokawa et al. 2005). Similar c-Fos transcript responses also were found in teleosts exposed to different types of stressors. In rainbow trout brain, the administration of kainic acid increased c-Fos mRNA expression after 60 min and for up to 120 min, at which time it then decreased towards control levels (Matsuoka et al. 1998). Another study described an increased c-Fos expression in brainstem of rainbow trout following a startle response (Bosch et al. 2001), and recently Salierno et al. (2006) found altered c-Fos protein expression in killifish which were exposed to harmful algal bloom toxins. However, no studies to date have investigated alterations in c-Fos mRNA or protein expression in brain of fish subjected to hypercapnic stress. Our results might suggest a central CO_2/pH-chemoreceptor activity in fish, too, as in higher vertebrates, where it is known that a chemoreceptor area in brain is involved in mediating a ventilator response to hypercapnia challenge by up regulating c-Fos gene expression.

In contrast to c-Fos, following acute hypercapnia, sea bass showed an evident downregulation of NHE-1 mRNA in both gill and kidney. These results are in contrast with previous studies conducted on killifish in which exposure to 1 % CO_2 for 1 h increased expression levels of NHE-1 protein in gill tissue (Edwards et al. 2005); however, they are in line with observation by Claiborne et al. (1999). They noted a decrease in expression of NHE-1-like proteins in the gills of *M. octodecimspinosus* after acidosis and postulated that such a decrease, presuming a basolateral location of these exchangers, would improve the efficiency of systemic net acid excretion. Instead, the slow increase in NHE-1 mRNA in sea bass kidney during chronic hypercapnia (96 h) was consistent with results of studies in mammals that showed an increase in NHE-1 messenger during chronic metabolic acidosis in the kidney cortex (Soleimani et al. 1995). Moreover, a recent scientific publication reported a significant increase in acid extruder protein levels (among which is NHE-1) and a decrease in acid loader proteins in kidney, brain, and heart of mice exposed to chronic hypercapnia; This is considered an adaptive response to attenuate intracellular acidosis induced by hypercapnia in this animal (Kanaan et al. 2007).

In addition to the other hypoxia-related gene GLUT2, c-Fos and NHE-1 may be considered as possible candidate biomarkers of a hypercapnic stress response in fish.

Molecular cloning and expression analysis of some genes involved in the innate immune response

The endogenous antimicrobial peptides (AMPs) are an important part of the innate immune system in various animal species. They are even more important in fishes than in mammals, as fish rely more on their innate immune system (Hancock and Scott 2000). However, this may be not so clear nowadays, as new insights in mucosal immunity and IgT by Sunyer's group show new perspectives (Zhang et al. 2010). AMPs, which play an important role in the defence against microbial invasion, are promptly synthesized at low metabolic cost, easily stored in large amounts, and readily available shortly after an infection (Oren and Shai

1996). Their value in innate host defence lies in their broad spectrum of activity against micro-organisms, their low toxicity for eukaryotic cells, their ease of synthesis (which does not require specialized cells or tissues), and their rapid diffusion rates, enabling them to be mobilized quickly to wounded or infected sites (for a review, see Boman 1995). Most AMPs molecules are amphiphilic and contain both cationic and hydrophobic surfaces, enabling them to insert into biological membranes (Douglas et al. 2003). Microbial killing is a consequence of the interaction of the AMP with the microbial outer membrane, which destabilizes the membrane and promotes channel formation. It is still not known whether channel formation alone promotes leakage of cytoplasmic contents, resulting in death of the organism, or whether introducing AMPs into the cytoplasm and interaction with cellular components also play a role in microbial killing (Lauth et al. 2002). Although the primary mode of action of antimicrobial peptides has been described as lysis of pathogens, they also have been reported to modulate gene expression in mouse macrophages (Scott et al. 2000). In addition, they might exert similar effects in fish macrophages or hepatocytes.

Several AMPs have been isolated in teleostean fishes such as misgurin in loach (*Misgurnus anguillicaudatus*) (Park et al. 1997), epinecidin in grouper (*Epinephelus coioides*) (Yin et al. 2005), pleurocidin in winter flounder (*P.americanus*) (Cole et al. 1997), moronecidin in hybrid striped bass (Lauth et al. 2002), pardaxin in sole (*Pardachirus marmoratus*) (Adermann et al. 1998), hepcidin in winter flounder (*P.americanus*), Atlantic salmon (*Salmo salar*), and perch (*P.fluviatilis*) (Douglas et al. 2003; Rossi et al. 2007), parasin in catfish (*Parasilurus asotus*) (Park et al. 1998), and dicentracin in sea bass (*D.labrax*) (Salerno et al. 2007). In fishes, AMPs usually are associated with peripheral blood leucocytes, mucosal surfaces and head kidney, which represents the primary organ involved in immune function (Robert et al. 1999; Smith et al. 2000; Sarmento et al. 2004). However, up to now, no studies have been conducted in fishes to investigate changes in the expression of genes that encode such immunologically relevant proteins as AMPs.

On the other hand, concern over the use of dietary antibiotics in aquaculture has encouraged industry to search for alternatives that both enhance performance and afford protection from disease. Mannans and glucans, derived from the outer cell wall of a specific strain of yeast *Saccharomyces cerevisiae*, seem to fit these criteria. Mannans and glucans have been shown to inhibit pathogen colonization by blocking type-1 fimbriae, filaments that allow pathogens to attach to the intestinal lining (Dawson and Pirvulescu 1999), to serve as an immune modulator, reducing intestinal microbial populations, and to improve the integrity and morphology of the intestinal mucosa.

The theoretical role of mannans and glucans in the gut has not been proven, but including these products in the diet has had a positive impact on animal performance. The efficacy of mannans and glucans as a growth promoter and immune response modulator has been shown in pigs (Miguel et al. 2002) and in a broad number of poultry species (Sonmez and Eren 1999; Iji et al. 2001). Few studies have examined the effect of these pronutrients on performance and the nonspecific (innate) immune system of fishes. However, in these studies, feeding mannans and glucans-supplemented diets to common carp (*C.carpio*) enhanced growth and significantly improved feed utilization, antibody levels, bactericidal and lysozyme activity, and alternative complement pathway activity (Staykov et al. 2005). In sea bass (*D.labrax*), incorporating mannans and glucans in the diet enhanced growth, activated immune response, and increased resistance to bacterial infection (Torrecillas et al. 2007; Terova et al. 2009b).

These considerations prompted our interest in studying the impact of a mannans and glucans-supplemented diet on the mRNA copy number of the AMP dicentracin in sea bass (*D.labrax*), with the aim to relate these expression levels to the quantity of the immune response modulator incorporated in the diet.

For the experiment, three diets based in a commercial pellet were reformulated to contain 0 ‰ (Control), 3 ‰ (MG3), or 5 ‰ (MG5) mannans-glucans, replacing standard carbohydrates. Sea bass (*D.labrax*) of about 280 g were stocked into three tanks connected to a water recirculation system where salinity was 22 g/L. Fish in the first tank were fed with the control diet MG0 (0 ‰ mannans-glucans), whereas fish in the other two tanks were fed with MG3 (3 ‰ mannans-glucans) or MG5 (5 ‰ mannans-glucans) diets, respectively. All fish were fed once a day at 1 % body weight/day. Fish from each of the three experimental groups were sampled after 30 and 60 days of

feeding. For the molecular biology analysis, head kidneys were isolated.

The mRNA copy numbers of dicentracin in the head kidney in response to different levels of mannans and glucans dietary supplementation are presented in Fig. 8. These data demonstrated that dicentracin gene expression was induced by mannans and glucans. Indeed, 30 days of feeding fish with diets containing mannans and glucans supplemented at either 3 or 5 ‰ significantly increased the dicentracin mRNA copy number as compared to the controls. Furthermore, the copy number in fish fed at 3 ‰ was significantly higher than that of the group fed at 5 ‰ for the same period of feeding mannans and glucans.

A longer feeding period (60 days) did not further increase the dicentracin mRNA copy number relative to the values recorded after 30 days of feeding in either the groups MG3 or the MG5. However, the transcript levels in fish fed at 3 ‰ proved to be significantly higher than those of the controls (Fig. 8).

Knowing that specific feed supplements can influence the intestinal microflora and modulate the immune response is a powerful tool to formulate feed rations without antibiotics. The use of mannans and glucans in terrestrial animals has been well documented in numerous trials, but only recently was their effectiveness in aquaculture also established. The effects of mannans and glucans on improving immune parameters have been reported in common carp (Staykov et al. 2005; Culjak et al. 2006), rainbow trout (Staykov et al. 2007), and European catfish (Bogut et al. 2006). The same effects also were observed for marine fish species. In the studies of Torrecillas et al. (2007), the immune function improved after incorporating mannans and glucans in the diet of sea bass juveniles. Immune parameters, phagocytic activity of leukocytes, and bacterial activity of the sera in the mannans and glucans-fed sea bass groups showed significantly improved dose response when compared to the control group. Disease resistance to bacterial infection, both by cohabitative challenge and by direct inoculation in the gut, also were enhanced when mannans and glucans were incorporated in the diets. In cohabitation trials, the presence of *Vibrio alginolyticus* on the head kidney of sea bass was 33 % for the control group and 8 and 0 %, respectively, for the 0.2 and 0.4 % mannans and glucans-fed groups.

The most exciting aspect of this study was the impact of mannans and glucans on an antimicrobial peptide gene expression. Mannans and glucans were active in upregulating dicentracin mRNA copy number in the head kidney of sea bass. The magnitude of transcript response was much higher in sea bass fed mannans and glucans at 3 ‰ than in those fed at 5 ‰. We recognize that dicentracin mRNA levels in our study do not measure physiological effects produced by the protein. Due to this, our hypothesis that mannans and glucans are an important trigger of the innate immune response in the sea bass is preliminary and will have to be confirmed in the future.

In conclusion, the assays developed and the baseline information now available would be useful for monitoring the occurrence and response to stress in cultured fish and possibly also for molecular marker-assisted selection of broodstock that respond to stress in appropriate ways.

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